

Some Effects of Hydrolytic Enzymes on Coupled and Uncoupled Electron Flow in Chloroplasts¹

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ABSTRACT

Digestion of spinach chloroplasts with pancreatic lipase or trypsin effectively uncoupled electron transport. Continued digestion led to inhibition of saturated rates of Hill reaction activity and a decrease in quantum yield. Irradiation with ultraviolet light decreased the quantum yield and inhibited Hill activity, but did not uncouple. Ascorbate-dichlorophenol-indophenol-mediated reduction of nicotinamide adenine dinucleotide phosphate was not appreciably inhibited by treatment with either of the enzymes or by ultraviolet irradiation.

Carbonylcyanide *m*-chlorophenylhydrazone became a potent inhibitor of electron transport after trypsin treatment of chloroplasts. It also inhibited, rather than uncoupled, electron transport in glutaraldehyde-fixed chloroplasts. No other uncouplers tested showed these effects. Glutaraldehyde fixation of chloroplasts also greatly reduced the inhibitory effects of lipase and trypsin digestion but not the inhibition by ultraviolet irradiation.

The inhibitory effects of trypsin and pancreatic lipase, and probably ultraviolet irradiation as well, appear to be due to a general breakdown of the membrane structure rather than inactivation of specific sites in the electron transport chain.

It is now well known that the light reactions of photosynthesis occur in the membrane structure of the chloroplast (21). The proposal of the chemiosmotic hypothesis (13, 14) for the production of ATP has led to increased interest in the integrity of the membrane structure as a vital factor in efficient operation of the photosynthetic process. Since the lamellar membranes are about 50% protein and 50% lipid (8), treatments which alter or destroy proteins and lipids might be useful in determining the role and importance of these substances in maintaining over-all membrane integrity and photosynthetic activity. Various proteolytic and lipolytic enzymes have been used in studies of the Hill reaction, photophosphorylation, fluorescence, and the morphology of the grana (2, 4, 5, 12, 15, 16).

In general, both proteolytic and lipolytic enzymes affect the system II reactions to varying degrees but have little effect on system I reduction of NADP with artificial electron donors (15). Digestion of *Euglena* chloroplasts with trypsin or papain has

been shown to disrupt drastically the lamellar membranes while treatment with wheat lipase appeared to separate the lamellae without substantially altering the membrane integrity. These observations were made by absorption microscopy (4).

Irradiation with ultraviolet light inhibits chloroplast reactions (7, 10, 11, 19, 20) in a manner similar to that with hydrolytic enzymes. It has been suggested that UV³ irradiation also inhibits by disrupting the membrane structure (11).

In the present study, the effects of porcine pancreatic lipase, trypsin, and UV irradiation on coupled and basal electron transport were measured. The probable mode of action of these treatments is discussed.

MATERIALS AND METHODS

Chloroplasts were prepared from spinach (*Spinacia oleracea*) obtained at a local market. After the leaves had been blended for 15 sec, the resulting homogenate was filtered through four layers of cheesecloth and centrifuged at 500g for 5 min to remove debris. The supernatant was centrifuged for 5 min at 5000g, and the pellet was washed in isolating medium (0.4 M sucrose, 0.015 M KCl, 0.05 M tricine, pH 7.6) and recentrifuged as above. The final pellet, consisting of chloroplasts and chloroplast fragments, was resuspended in isolating medium or phosphate buffer at a chlorophyll concentration of about 0.5 mg/ml and is termed chloroplasts. Glutaraldehyde fixation of spinach chloroplasts was carried out according to the method of Park *et al.* (18).

Porcine pancreatic lipase, type II (Sigma Chemical Co., St. Louis, Missouri), was further purified by dissolving 400 mg in 100 ml of 0.015 M phosphate buffer, pH 6.5, and centrifuging at 25,000g for 10 min. The clear supernatant was then taken to 50% saturation with solid ammonium sulfate and centrifuged as above. The resulting pellet was redissolved in 0.015 M phosphate buffer (pH 6.5), divided into convenient aliquots, and frozen until used. Twice recrystallized trypsin (Nutritional Biochemicals, Cleveland, Ohio, or Sigma Chemical Co.) was normally dissolved in isolating medium or phosphate buffer before addition to the sample. In a typical experiment, 40 µg of trypsin per mg of chlorophyll gave total inhibition of DCIP reduction in about 30 min at 25 C. UV irradiation (2537 Å) was performed as previously described (10).

Enzyme incubations were carried out in 50-ml Erlenmeyer flasks suspended in a water bath at 25 C with continuous shaking. Samples were taken at various times and immediately diluted 5-fold with ice-cold suspending medium in order to retard the enzyme digestion while the assays were being made.

DCIP reduction was assayed at 610 nm and NADP reduction at 340 nm in a Beckman DU spectrophotometer. The reaction times varied between 15 sec and 3 min, depending on the activ-

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³ Abbreviations: UV: ultraviolet; DCIP: dichlorophenolindophenol; CCCP: carbonylcyanide *m*-chlorophenylhydrazone; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS: phenazine methosulfate.

ity. DCIP was dissolved in water, refrigerated, and used within 2 days. Actinic light, provided by a 500-w projection lamp, was passed through a 5-cm water filter and an infrared-absorbing filter (Balzer Calflex C) and focused on a 1-cm path length cuvette. The light intensity at the surface of the cuvette was about 7.2×10^5 ergs $\text{cm}^{-2} \text{sec}^{-1}$. For measuring intensity curves, actinic light at 651 nm was provided by a 650-w Sun-Gun lamp in conjunction with a 651 nm Balzer interference filter (half-band width 11 nm), an infrared-absorbing filter, and a 5-cm water filter.

Chlorophyll was measured by the method of Arnon (1). Fatty acids were determined by extracting the lipids from chloroplasts with ether, dissolving in ethanol, and titrating with KOH (3). A pH of 9.5 was used as the end point of the titration.

RESULTS AND DISCUSSION

Effects on Quantum Yield. At least part of the UV inactivation of photosynthesis probably is due to disruption of the photochemistry in the reaction centers of system II since UV irradiation of spinach chloroplasts decreases the variable yield fluorescence at the same rate as DCIP reduction under saturating actinic intensities (11). To substantiate further the hypothesis that the reaction centers are affected, experiments were performed measuring the relative quantum yield after various periods of UV irradiation. In the $(\text{rate})^{-1}$ against $(\text{intensity})^{-1}$ plot shown in Figure 1, quantum yield is directly related to the slope of the lines and the intercept is equal to $(\text{saturation rate})^{-1}$. The results depicted in Figure 1A show that UV irradiation indeed decreases the quantum yield as well as the saturation rate. Figures 1B and 1C show that treatment of chloroplasts with pancreatic lipase or trypsin decreased the relative quantum yield of DCIP reduction. The light-saturated rates were also decreased at about the same rate (but see below), analogous to UV inactivation. The decrease in both quantum yield and light-saturated rate of DCIP reduction suggests that both photochemical and enzymatic reactions are being affected.

Effects on Coupling. Studies by Gressel and Avron (5) showed that photophosphorylation with PMS was inhibited to varying

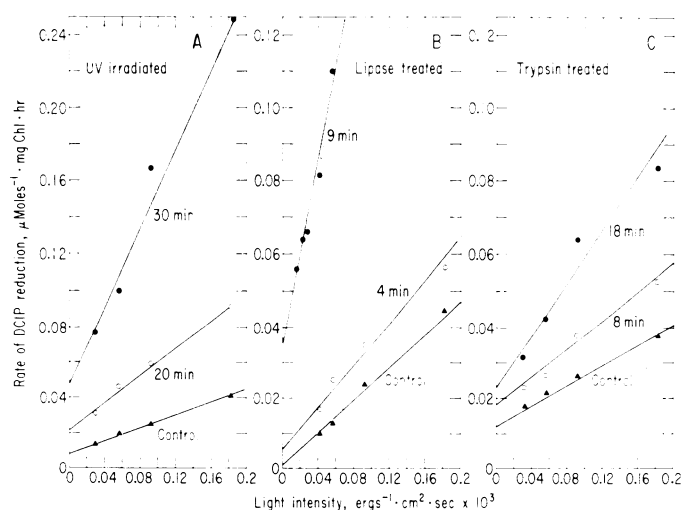


FIG. 1. Effects on relative quantum yield and light-saturated rate of DCIP reduction after UV irradiation (A), pancreatic lipase digestion (B), and trypsin digestion (C). Reaction mixtures consisted of: sucrose, 0.4 M; KCl, 15 mM; tricine, 50 mM (pH 7.6); DCIP, 33 μM ; and chloroplasts containing about 40 μg of chlorophyll, the exact amount varying slightly from experiment to experiment. The total volume was 3.0 ml. UV irradiation and enzyme digestion as in "Materials and Methods."

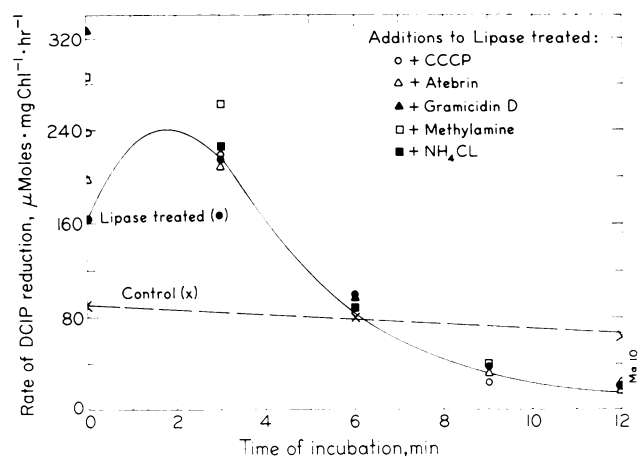


FIG. 2. Effect of uncoupling agents on DCIP reduction under light-saturating conditions after digestion with pancreatic lipase. Reaction conditions as in Figure 1 with uncouplers added to give the following concentrations: CCCP, 3.3 μM ; atebrin, 10 μM ; NH_4Cl , 1 mM; methylamine-HCl, 20 mM; and gramicidin D, 0.5 $\mu\text{g}/\text{ml}$. Solid line shows lipase-treated sample in absence of added uncouplers. Dashed line shows control sample without added uncouplers. The shape of the curve during the first 3 min is uncertain because of practical difficulties in taking samples at intervals of less than about 3 min. Note that considerable uncoupling had occurred during the time between addition of the enzyme and removal of the zero time sample (about 30 sec).

degrees by different lipases. PMS photophosphorylation is also inhibited by UV irradiation (11, 19, 20), the degree of inhibition apparently being related to the intensity of the actinic light used (11). To explore the possibility that the coupled and basal electron transport systems may be situated at separate locations in the lamellar membranes and hence possibly be affected differently by these treatments, a series of experiments was performed comparing the effects of uncouplers on chloroplasts treated with either UV irradiation, pancreatic lipase, or trypsin.

Figure 2 shows the results of an experiment measuring the effects of lipase on coupled and basal electron transport under saturating actinic intensities. Under the conditions employed, the enzyme itself effectively uncoupled chloroplasts after 2 to 3 min of incubation. Continued incubation caused a decrease in photo-reductive activity which paralleled the decrease in quantum yield (Fig. 1B). This uncoupling reaction by lipase apparently is complete because addition of chemical uncouplers did not cause any further increase in the electron transport rate (Fig. 2).

Trypsin also showed a rapid uncoupling effect although it did not appear to uncouple as effectively as lipase. As shown in Figure 3A, the effects were qualitatively similar to the lipase treatment although addition of methylamine-HCl (also atebrin, gramicidin D, or ammonium chloride) gave an additional increase in electron flow.

One uncoupler, CCCP, gave strikingly different results. As the trypsin digestion proceeded, addition of CCCP caused a pronounced inhibition of electron transport (Fig. 3B). The degree of inhibition increased as the period of incubation increased. The initial phase of the inhibition is first order with respect to incubation time and the kinetics do not follow that of either the uncoupling or inhibition reactions caused by the trypsin itself. The CCCP concentration (3.3 μM) was not high enough to cause the inhibition. This inhibition by CCCP is discussed in more detail elsewhere (9). In contrast, addition of CCCP to either lipase-treated (Fig. 2) or UV-irradiated chloroplasts (Fig. 4) enhanced electron transport, although in UV-irradiated chloroplasts CCCP showed a slight inhibition as irradi-

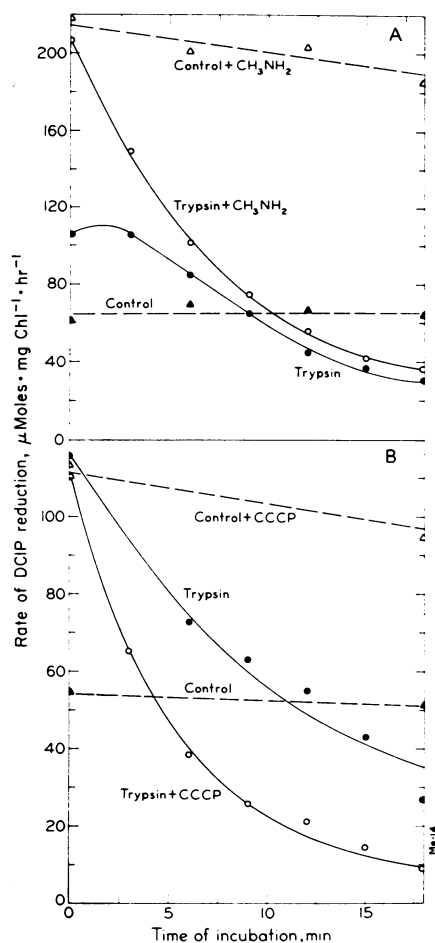


FIG. 3. Effects of methylamine-HCl (A) and CCCP (B) on DCIP reduction after trypsin digestion. Reaction conditions as in Figures 1 and 2. As with lipase treatment, considerable uncoupling occurred between the time of addition of the enzyme and removal of zero time sample. CCCP concentration ($3.3 \mu\text{M}$) was less than optimal for complete uncoupling.

ation time increased. This can be seen by the slightly increased slope of the +CCCP curve in Figure 4.

UV irradiation, unlike lipase and trypsin treatment, did not show any uncoupling effects and the kinetics of the inhibition of basal and coupled electron transport rates were the same (compare control and + CH_3NH_2 curves in Fig. 4).

The uncoupling action exhibited by lipase and trypsin could be expected since uncoupling can be considered to be a disruption of the lamellar membranes in some manner allowing a proton or potential gradient to be dissipated, or by preventing its formation. Lamellar membranes are known to consist of about 50% protein and 50% lipid (8). Destruction or modification of either the protein or lipid components of the membrane could be expected to alter the membrane's permeability to protons or other ions, thus explaining the uncoupling action observed.

As reported earlier for UV-irradiated and lipase-treated chloroplasts (7, 10, 15, 20), trypsin-treated chloroplasts show unimpaired rates of NADP reduction with ascorbate-DCIP as electron donor.

Effects on Glutaraldehyde-fixed Chloroplasts. Glutaraldehyde fixation of chloroplasts tends to stabilize system II activity (18). Although such chloroplasts have rather low initial photo-reductive capacity, this activity remains relatively constant over a period of several days at 0 C. Glutaraldehyde is known to cross-link proteins, both intra- and intermolecularly, without greatly affecting their conformation or activity (6). Therefore, the possi-

bility was tested that glutaraldehyde fixation would stabilize the membranes against the action of lipase, trypsin, and UV irradiation.

Figure 5A shows results of an experiment comparing the effects of lipase on fixed and unfixed chloroplasts. As shown in the

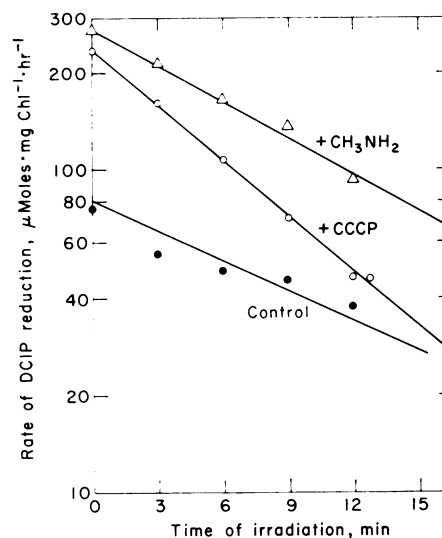


FIG. 4. Effect of uncoupling agents on DCIP reduction after UV irradiation. Reaction mixture and uncoupler concentrations as in Figures 1 and 2.

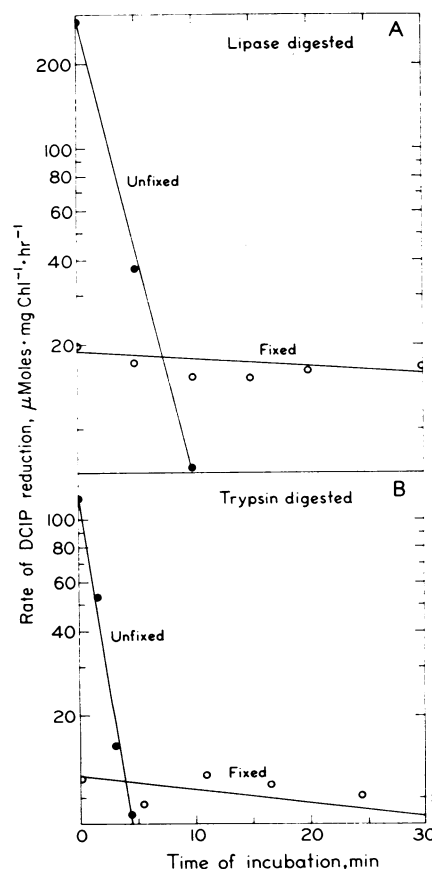


FIG. 5. Effect of pancreatic lipase digestion (A) and trypsin digestion (B) on DCIP reduction in normal and glutaraldehyde-fixed chloroplasts. Reaction mixture consisted of: phosphate buffer, 0.01 M (pH 7.3); DCIP, $33 \mu\text{M}$; and chloroplasts containing $40 \mu\text{g}$ of chlorophyll.

figure, fixation offered striking protection against the effects of lipase on DCIP reduction. The very large difference in initial activities between fixed and unfixed chloroplasts is due to the uncoupling effect of lipase on the unfixed material discussed above. Although ATP was not measured in fixed chloroplasts, data from other laboratories (17, 18) have shown that fixed chloroplasts retain light-induced proton uptake activity and show a stimulation of electron transport in the presence of chemical uncouplers. These results suggest that in fixed chloroplasts the phosphorylation mechanism is at least partially intact. Experiments comparing fixed and unfixed chloroplasts were run in dilute phosphate buffer rather than the usual sucrose medium because fixed chloroplasts were nearly inactive when suspended in sucrose. To insure identical conditions in each experiment, both types of chloroplasts were suspended in buffer only, and the incubation times were kept as short as practical to avoid large activity losses due to the suspending medium. The enzyme concentrations were adjusted to inhibit DCIP reduction totally in unfixed chloroplasts after about 10 to 12 min of incubation. The inhibition curves for fixed chloroplasts are corrected for losses of activity in the control sample which amounted to about 20 to 30% after 30 min of incubation.

Titration of the fatty acid (3) liberated after lipase treatment in both fixed and unfixed chloroplasts did not reveal any great difference in lipase activity. This difference in amount of fatty acid liberated from fixed and unfixed chloroplasts incubated under identical conditions was less than a factor of 2 while the difference in the rates of inhibition of DCIP reduction was greater than a factor of 10 (Fig. 5A). It thus appears that the protection afforded by glutaraldehyde fixation was not due simply to impairment of the lipase action.

Figure 5B shows that fixation also protected chloroplasts against the action of trypsin. Trypsin still appeared to partially uncouple fixed chloroplasts in a manner similar to unfixed material, but relatively little decrease in photoreduction was evident, even after extended periods of incubation. Surprisingly, CCCP (3.3 or 1.7 μ M) did not uncouple fixed chloroplasts but rather acted as an inhibitor of electron flow comparable to its effect after trypsin treatment of unfixed chloroplasts. As in the lipase experiments, the data in Figure 5B are corrected for losses of activity in the control chloroplasts.

In the fixation procedure employed (18), whole spinach leaves were fixed with glutaraldehyde by vacuum infiltration and the chloroplasts were then isolated from this material. This procedure gave preparations consisting almost entirely of whole chloroplasts while the unfixed material contained many fragments and relatively few whole chloroplasts. To be sure that the presence of the outer chloroplast membrane was not affecting the ability of the enzymes to act on the lamellae, a preparation of whole, unfixed chloroplasts was made. Lipase acted in a manner identical to that observed with fragments. Fixed chloroplasts were thoroughly washed (4 times with distilled water) to insure that residual free glutaraldehyde was not interfering with the enzyme activity. Thus it appears that it was indeed the fixation itself which was protecting the chloroplasts from the action of both trypsin and lipase.

In contrast to the results with lipase and trypsin, fixation did not offer any protection against the action of UV irradiation.

The enzymes used in these studies were commercial preparations. The pancreatic lipase was further purified (see "Materials and Methods") and twice recrystallized trypsin from two different sources gave identical results, but the purity of the lipase with respect to proteolytic activity (and vice versa) was not deter-

mined. However, the data presented above indicate that this type of contamination was not a factor. For example, although both enzymes uncoupled electron transport, the degree of uncoupling was quite different and the effects of CCCP on the two systems were also totally different.

The data presented in this paper support the notion that both lipase and trypsin inhibit by effecting a general breakdown of the lamellar structure rather than by attacking some specific component, or components, in the electron transport and phosphorylation mechanism. UV irradiation seems to have a somewhat more specific mode of action than either lipase or trypsin although it too inhibits a broad spectrum of reactions associated with photosynthesis. UV irradiation probably also acts by disrupting some structural pattern in the membrane and not by the destruction of a single component.

These three treatments may prove useful as tools for further examining the relationships between membrane structural integrity and biochemical activity.

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